Chapter 6
Microscopic Examination

The microscopic portion of this procedure depends on very sophisticated optics. Without proper alignment and adjustment, the microscope will not function at peak efficiency. Therefore, it is imperative that all portions of the microscope, from the light sources to the oculars, are adjusted properly. These adjustments should be practiced until they become second nature. The procedures for adjusting the epifluorescent mercury bulb and the transmitted light filament are presented in Appendix B.

Adjustment of Interpupillary Distance and Oculars for Each Eye

These adjustments are necessary so that eye strain is reduced to a minimum. These adjustments must be made for each individual using the microscope. This section assumes the use of a binocular microscope.

Interpupillary Distance

The spacing between the eyes varies from person to person and must be adjusted for each individual using the microscope.

Step 1 Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

Step 2

Using both hands, adjust the oculars in and out until a single circle of light is observed while looking through the two oculars with both eyes.

Ocular Adjustment for Each Eye.

This section assumes a focusing ocular(s). This adjustment can be made two ways, depending upon whether or not the microscope is capable of photomicrography and whether it is equipped with a photographic frame which can be seen through the binoculars.

!

Persons with astigmatic eyes should always wear their contact lenses or glasses when using the microscope.

For microscopes not capable of photomicrography

This section assumes only the right ocular is capable of adjustment.

Step 1

Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

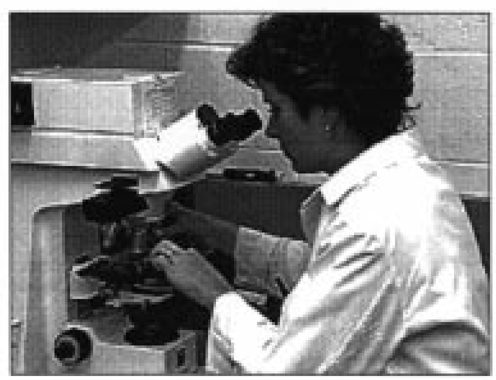
Step 2 Place a card between the right ocular and eye, keeping both eyes open. Using the fine adjustment, focus the image for the left eye to its sharpest point.

Step 3 Now transfer the card to between the left eye and ocular. **Without touching the coarse or fine adjustment,** and with keeping both eyes open, bring the image for the right eye into sharp focus by adjusting the ocular collar at the top of the ocular.

For microscopes capable of viewing a photographic frame through the viewing binoculars

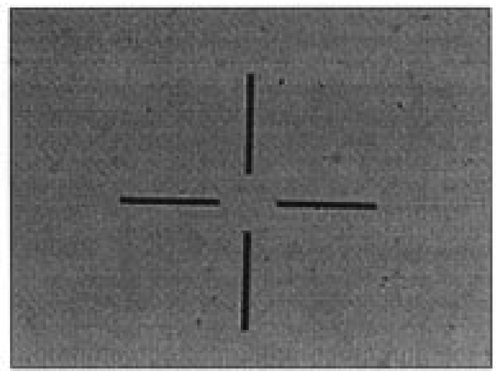
This section assumes both oculars are adjustable.

Step 1 Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.



Step 2 After activating the photographic frame, place a card between the right ocular and eye, keeping both eyes open. Using the correction (focusing) collar on the left ocular, focus the left ocular until the double lines in the center of the frame are as sharply focused as possible.

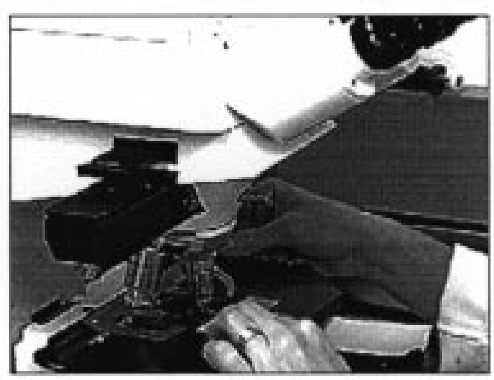
Step 3 Now transfer the card to between the left eye and ocular. Again keeping both eyes open, bring the image of the double lines in the center of the photographic frame into as sharp a focus for the right eye as possible by adjusting the ocular correction (focusing) collar at the top of the right ocular.



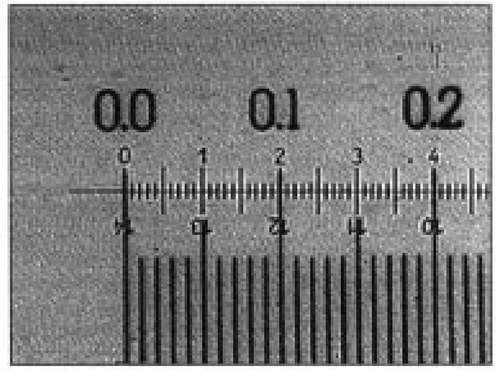
Calibration of an Ocular Micrometer[1]

This section assumes that an ocular reticle has been installed in one of the oculars by a microscopy specialist and that a stage micrometer is available for calibrating the ocular micrometer (reticle). Once installed the ocular reticle should be left in place. The more an ocular is manipulated the greater the probability is for it to become contaminated with dust particles. This calibration should be done for each objective in use on the microscope. If there is an optivar[2] on the microscope, then the calibration procedure must be done for the respective objective at each optivar setting.

Step 1 Place the stage micrometer on the microscope stage, turn on the transmitted light, and focus the micrometer image using the coarse and fine adjustment knobs for the objective to be calibrated. Continue adjusting the focus on the stage micrometer so you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.



Step 2 Adjust the alignment of the stage and ocular micrometers so the 0 line on the ocular micrometer is exactly superimposed on the 0 line on the stage micrometer.



Step 3 Without changing the stage adjustment, find a point as distant as possible from the two 0 lines where two other lines are exactly superimposed.

Step 4 Determine the number of ocular micrometer spaces as well as the number of millimeters on the stage micrometer between the two points of superimposition. For example: Suppose 48 ocular micrometer spaces equal 0.6 mm.

Step 5 Calculate the number of mm/ocular micrometer space.

Calculating Ocular Micrometer Space

$$\frac{\text{0.6 mm}}{\text{48 o.m. spaces}} = \text{0.0125 mm/o.m. space}$$

Step 6 Since most measurements of microorganisms are given in µm rather than mm, the value calculated above must be converted to µm by multiplying it by 1,000 µm/mm.

Calculating Ocular Micrometer Space

$$\frac{\text{0.6 mm}}{\text{48 o.m. spaces}} = \text{0.0125 mm/o.m. space}$$

0.0125 mm/o.m. space x 1,000 µm/mm
= 12.5 µm/o.m. space

Step 7 Follow steps 1 through 6 for each objective. It is helpful to record this information in a table, like the example at right, which can be kept near the microscope.

Item #	Objective Power	Description	No. of Ocular Micrometer Spaces	No. of Stage Micrometer millimeters[a]	µm/Ocular Micrometer Space[b]
1	10X	N.A.[c] =			
2	20X	N.A. =			
3	40X	N.A. =			
4	100X	N.A. =			

[a] 1,000 µm/mm

[b] (Stage Micrometer length in mm x (1,000 µm/mm)) ÷ No. Ocular Micrometer Spaces

[c] N.A. stands for numerical aperture. The numerical aperture value is engraved on the barrel of the objective.

Köhler Illumination

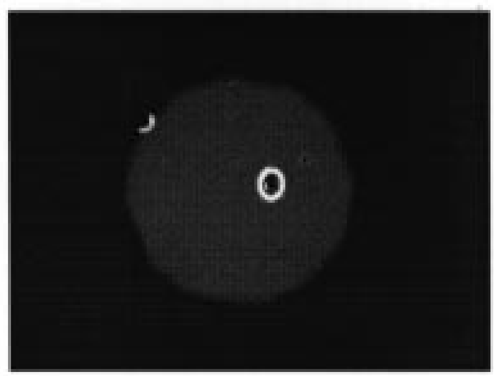
This section assumes that Köhler illumination will be established for only the 100X oil D.I.C. or Hoffman modulation® objective, which will be used to identify internal morphological characteristics in *Giardia* cysts and *Cryptosporidium* oocysts. If by chance more than one objective is to be used for either D.I.C. or Hoffman modulation® optics, then each time the objective is changed, Köhler illumination must be re-established for the new objective lens. Previous sections have adjusted oculars and light sources. This section aligns and focuses the light going through the condenser underneath the stage at the specimen to be observed. If Köhler illumination is not properly established, then D.I.C. or Hoffman modulation® optics will not work to their maximal potential. These steps need to become second nature and must be practiced regularly until they are a matter of reflex rather than a chore.

Step 1 Place a prepared slide on the microscope stage, place oil on the slide, move the 100X oil objective into place, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

Step 2 At this point both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Now close down the radiant field diaphragm in the microscope base until the lighted field is reduced to a small opening.

Step 3 Using the condenser centering screws on the front right and left of the condenser, move the small lighted portion of the field to the center of the visual field.

Step 4 Now look to see whether the leaves of the iris field diaphragm are sharply defined (focused) or not.



If they are not sharply defined, they can be focused distinctly by changing the height of the condenser up and down with the condenser focusing knob while you are looking through the binoculars. Once you have accomplished the precise focusing of the radiant field diaphragm leaves, open the radiant field diaphragm until the leaves just disappear from view.

Step 5 The aperture diaphragm of the condenser should be adjusted now to make it compatible with the total numerical aperture of the optical system. This is done by removing an ocular, looking into the tube at the rear focal plane of the objective, and stopping down the aperture diaphragm iris leaves until they are visible just inside the rear plane of the objective.

Step 6 After completing the adjustment of the aperture diaphragm in the condenser, return the ocular to its tube and proceed with the adjustments required to establish either D.I.C. or Hoffman modulation® optics.

Microscopic Examination

Microscopic work performed by a single analyst should not exceed 4 hours/day nor more than 5 consecutive days/week. Intermittent rest periods during the 4 hours/day are encouraged.

Step 1 Remove the dry box from 4°C storage and allow it to warm to room temperature before opening.

Step 2 Adjust the microscope to ensure that the epifluorescence and Hoffman modulation® or D.I.C. optics are in optimal working order.

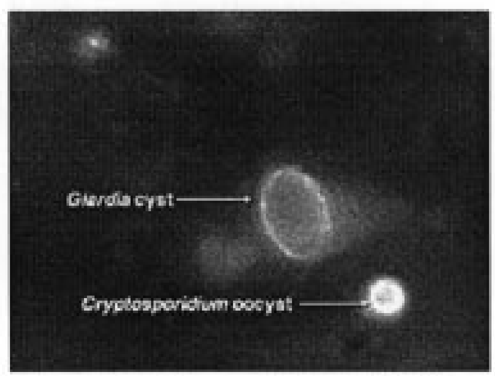
Make sure that the fluorescein isothiocyanate cube is in place in the epifluorescent portion of the microscope (see the section on Sample Examination below.) Detailed procedures required for adjusting and aligning the microscope are found in Appendix B.

Assay Controls

The purpose of these controls is to ensure that the assay reagents are functioning, that the assay procedures have been properly performed, and that the microscope has been adjusted and aligned properly.

Assay Giardia/Cryptosporidium Control

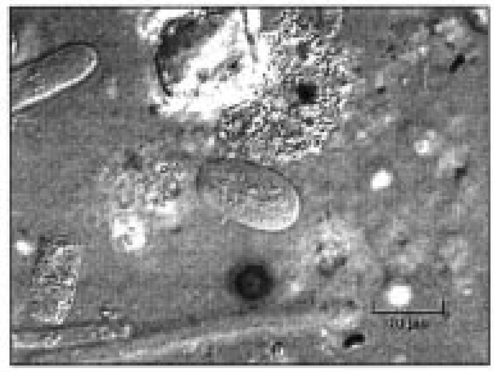
Step 1 Using epifluorescence, scan the positive control slide at no less than 200X total magnification for apple-green fluorescence of *Giardia* cyst and *Cryptosporidium* oocyst shapes. Background fluorescence of the membrane should be either very dim or nonexistent.



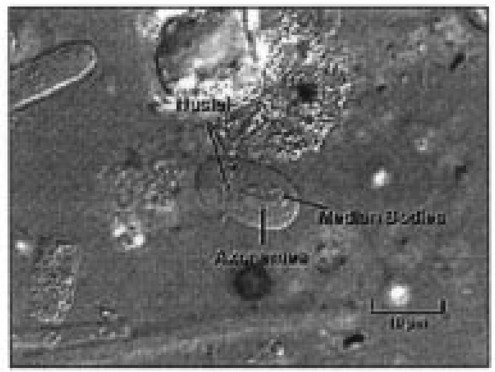
Cryptosporidium oocysts may or may not show evidence of oocyst wall folding, which is characterized under epifluorescence by greater concentrations of FITC along surface fold lines. Whether the oocysts show this characteristic depends on the manner in which the oocysts have been treated and the amount of turgidity they have been able to maintain[3].

! If no apple-green fluorescing *Giardia* cyst or *Cryptosporidium* oocyst shapes are observed, then the fluorescent staining did not work or the positive control cyst preparation was faulty. Do not examine the water sample slides for *Giardia* cysts and *Cryptosporidium* oocysts. Recheck reagents and procedures to determine the problem.

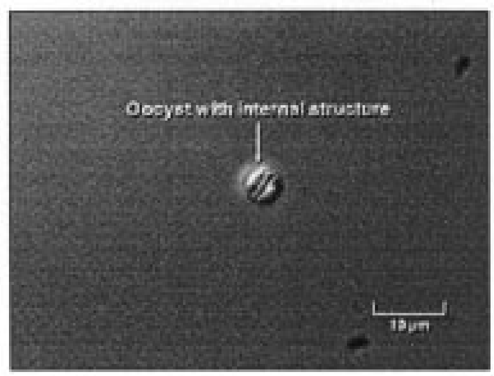
Step 2 If apple-green fluorescing cyst and oocyst shapes are observed, change the microscope from epifluorescence to the 100X oil immersion Hoffman modulation® or differential interference contrast objective.



At no less than 1,000X total oil immersion magnification, examine *Giardia* cyst shapes and *Cryptosporidium* oocyst shapes for internal morphology.



The *Giardia* cyst internal morphological characteristics include 1-4 nuclei, axonemes, and median bodies. *Giardia* cysts should be measured to the nearest 0.5 µm with a calibrated ocular micrometer. Record the length and width of cysts. Also record the morphological characteristics observed. Continue until at least 3 *Giardia* cysts have been detected and measured in this manner.



The *Cryptosporidium* oocyst internal morphological characteristics include 1-4 sporozoites. Examine the *Cryptosporidium* oocyst shapes for sporozoites and measure the oocyst diameter to the nearest 0.5 µm with a calibrated ocular micrometer. Record the size of the oocysts. Also record the number, if any, of sporozoites observed. Sometimes a single nucleus is observed per sporozoite. Continue until at least 3 oocysts have been detected and measured in this manner.

Assay Negative Control

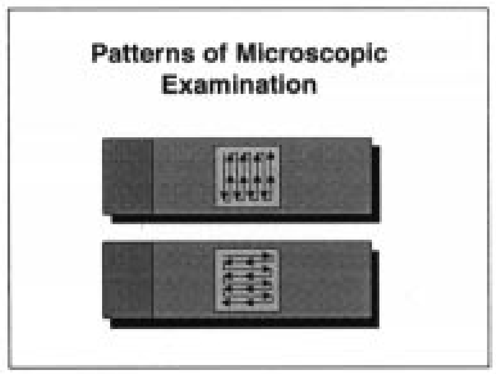
Step 1 Using epifluorescence, scan the negative control membrane at no less than 200X total magnification for apple-green fluorescence of *Giardia* cyst and *Cryptosporidium* oocyst shapes.

Step 2 If no apple-green fluorescing cyst or oocyst shapes are found, and if background fluorescence of the membrane is very dim or nonexistent, continue with examination of the water sample slides.

! If apple-green fluorescing cyst or oocyst shapes are found, discontinue examination since possible contamination of the other slides is indicated. Clean the equipment (see Appendix A), recheck the reagents and procedure, and repeat the assay using additional aliquots of the sample.

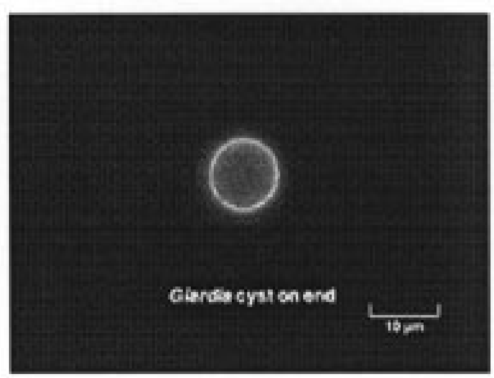
Sample Examination

Scan each slide in a systematic fashion, beginning with one edge of the mount and covering the entire coverslip. An up-and-down or a side-to-side scanning pattern may be used. The diagram at the right illustrates two alternative patterns for systematic slide scanning.

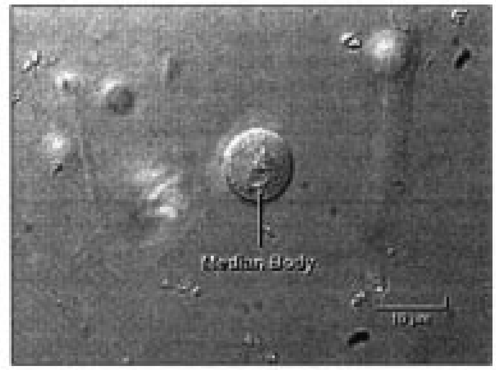


Empty Counts, Counts with Amorphous Structure, Counts with Internal Structure, and Total IFA Count

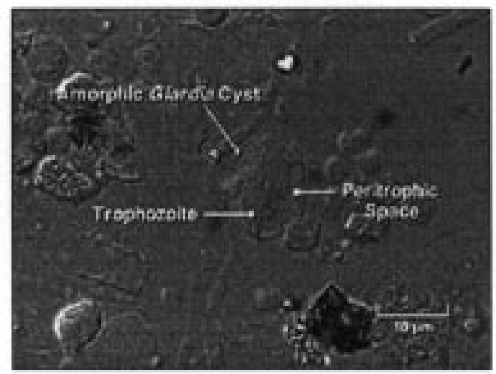
Step 1 When appropriate responses have been obtained for the positive and negative controls, use epifluorescence to scan the entire coverslip from each sample at not less than 200X total magnification for apple-green fluorescence of cyst and oocyst shapes.



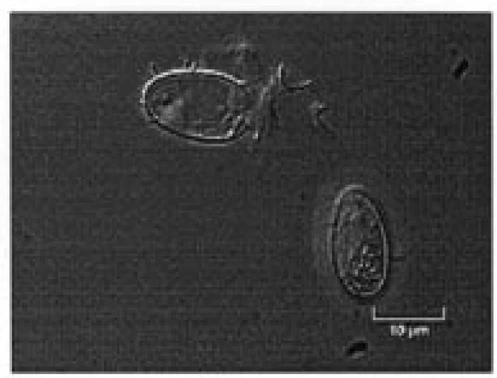
Step 2 When brilliant, apple-green fluorescing round-to-oval objects (8 to 18 μm long by 5 to 15 μm wide) with brightly highlighted edges are observed, switch the microscope to either Hoffman modulation® or D.I.C. optics.



Look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). If these atypical structures are not observed, then categorize such apple-green fluorescing objects of the aforementioned size and shape as either empty *Giardia* cysts, *Giardia* cysts with amorphous structure, or *Giardia* cysts with internal structures (nuclei, axonemes, and median bodies).



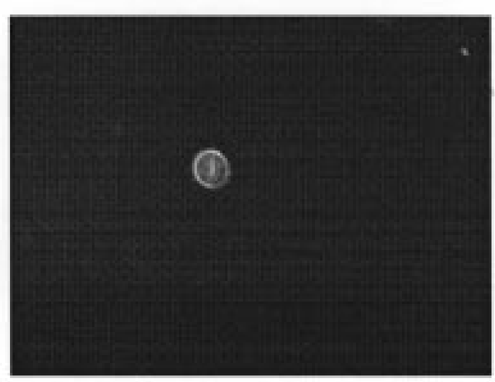
Record the shape and measurements (to the nearest 0.5 μm at 1,000X total magnification) for each such object. Record the internal structures observed.



Sum the counts of empty *Giardia* cysts, *Giardia* cysts with amorphous structure, and *Giardia* cysts with internal structures. Report this sum as the total *Giardia* IFA count (see Report Forms in Appendix zebbo).

! *Giardia* cysts with internal structures must be confirmed by a senior analyst.

Step 3 When brilliant, apple-green fluorescing ovoid or spherical objects (3 to 7 μm in diameter) with brightly highlighted edges are observed, switch the microscope to either Hoffman modulation® or D.I.C. optics. Look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). If these atypical structures are not observed, then categorize such apple-green fluorescing



objects of the aforementioned size and shape as either empty *Cryptosporidium* oocysts, *Cryptosporidium* oocysts with amorphous structure, or *Cryptosporidium* oocysts with internal structure (1 to 4 sporozoites/oocyst).

Although not a defining characteristic, surface oocyst folds may be observed in some specimens.

Record the shape and measurements (to the nearest 0.5 µm at 1,000X total magnification) for each such object. Record the number of sporozoites observed. Sum the counts of empty *Cryptosporidium* oocysts, *Cryptosporidium* oocysts with amorphous structure, and *Cryptosporidium* oocysts with internal structure. Report this sum as the total *Cryptosporidium* IFA count (see Appendix D, Sample Forms).

Cryptosporidium oocysts with sporozoites must be confirmed by a senior analyst.

End Notes

[1] Melvin, D.M. and M.M. Brooke. 1982. Laboratory Procedures for the Diagnosis of Intestinal Parasites. U.S. Department of Health and Human Services, HHS Publication No. (CDC) 82-8282.

[2] A device between the objectives and the oculars that is capable of adjusting the total magnification.